Immune Activation and Oxidative Damage in HIV-Positive and HIV-Negative Adolescents

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Summary: In a cross-sectional study involving subjects from the Reaching for Excellence in Adolescent Health cohort, we examined the associations between HIV status, disease severity, immune activation, and oxidative damage. Subjects (265 HIV-positive and 127 HIV-negative) were young (range: 14–23 years of age) and primarily female (75%) and black (67%). Many subjects, particularly female subjects, were overweight or obese. Relatively few HIV-positive subjects had advanced HIV disease (13%), and 54% were taking antiretroviral therapy (ART). The 2 markers of oxidative damage used in this study (plasma malondialdehyde and protein carbonyl concentrations) did not correlate with each other, and neither was higher in HIV-positive subjects than in HIV-negative controls. Increased oxidative damage was seen in association with male gender, cigarette smoking, marijuana use, immune activation (as indicated by activated $CD8^+$ T-cell counts and plasma C-reactive protein concentration), and use of ART, however. Plasma ceruloplasmin was associated with decreased oxidative damage in HIV-positive subjects, although this association was not seen in those taking ART.

Key Words: oxidative stress, HIV, adolescent, acute-phase response, C-reactive protein, ceruloplasmin, neopterin, female, black, Hispanic, obesity, malondialdehyde, protein carbonyl

(*J Acquir Immune Defic Syndr* 2005;38:180–190)

HIV and other chronic infections induce prolonged immune system activation that may cause local or systemic oxidative stress and thus result in oxidative damage. Oxidative stress occurs when the balance of antioxidant protection is overmatched by the production of free radicals, primarily reactive oxygen and nitrogen molecules, resulting in oxidative damage to proteins, lipids, and nucleic acids.[1] Free radicals are produced by normal physiologic processes, such as electron transport in mitochondria, and their production is particularly high in "activated" phagocytic cells that produce reactive oxygen as part of their microbicidal response. Thus, immune system activation may produce significant local or systemic oxidative stress. The amount of oxidative damage that results from this stress depends on the status of the antioxidant protection systems at the site of inflammation and in the organism as a whole.

Oxidative damage to cells of the immune system may impair the immune response to HIV and thus hasten the progression of HIV disease. Oxidative stress may also hasten progression of disease by enhancing HIV replication via nuclear factor (NF)-κB signaling.[2] Protecting against such damage and stress could slow the progression of HIV disease. In this cross-sectional study, we examined the relation of immune system activation to levels of oxidative damage in HIV-positive and -negative adolescents and young adults.

SUBJECTS AND METHODS

Study Population

The Reaching for Excellence in Adolescent Care and Health (REACH) study was a prospective observational cohort study on the progression of HIV infection in adolescents conducted at 15 clinical sites in the United States.[3,4] A standardized protocol was developed through the Adolescent Medicine HIV/AIDS Research Network. Between March 1996 and November 1999, 325 adolescents from 12 to 18 years of age who had acquired HIV infection through sexual activity or intravenous drug use were recruited. In addition, 171 HIV-negative adolescents were recruited from the same sites using selection criteria to make the HIV-negative and HIV-positive groups comparable with regard to risk behavior profiles and demographic characteristics (including age, gender, race, and ethnicity). This report describes a supplemental cross-sectional study focusing on measures of immune activation and oxidative damage that was conducted during study visits between January and October 2000. One site did not participate because of logistical difficulties. Among the 436 participants active in the 14 REACH network sites at the time of the dietary intake study, 391 agreed to participate (264 HIV-positive and 127 HIV-negative subjects). The study re-

Received for publication September 24, 2003; accepted July 7, 2004.

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Funded by National Institutes of Health grant R01 AI46183 and Agricultural Research Service (ARS) Project 5306-51530-006-00D. The Reaching for Excellence in Adolescent Health study was funded by grant U01-HD32830 from the National Institute of Child Health and Human Development with cofunding from the National Institute on Drug Abuse, Allergy and Infectious Disease, and the National Institute of Mental Health.

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ceived approval by the Human Subjects Review Boards at the University of California at Davis, Iowa State University, University of Alabama at Birmingham, and each clinic site. All participants provided informed written consent.

Data Collected by the Reaching for Excellence in Adolescent Care and Health Study

Data from the REACH study were collected by face-to-face interviews, interactive computer interviews (eg, for collection of data on drug and alcohol use), medical record abstractions, and physical and laboratory examinations. HIV-positive subjects were seen every three months, whereas HIV-negative subjects were seen every six months. A detailed description of the REACH study protocol is described elsewhere.[3,4]

Anthropometric Measurements

Participants were gowned and weighed at each visit using digital scales accurate to one-tenth decimal place. Heights were measured using calibrated stadiometers installed at each study site. Body mass index (BMI) was calculated for each participant as weight (kg)/height (m) squared.

HIV and Immune Activation Variables

Laboratory tests were performed at local clinic sites according to the REACH protocol described elsewhere.[3–6] Activated $CD8^+$ T cells were measured as described previously[7] using CD38 and D-related human leukocyte antigen (HLA-DR) as markers of activation. Absolute $CD4^+$ T-cell counts for HIV-positive participants were stratified based on Centers for Disease Control and Prevention (CDC) criteria for HIV/AIDS classification: $\geq$500, 200 through 499, and $<$200 cells/mm^3. Clinical progression was ranked (early/asymptomatic, intermediate/symptomatic, late/AIDS-indicator illnesses) using CDC guidelines.[8] Quantitative HIV-1 RNA viral load in plasma was measured in a centralized laboratory on frozen specimens using either nucleic acid sequence–based amplification (NASBA) or NucliSens assays (Organon Teknika, Durham, NC) as described elsewhere.[9]

Antiretroviral therapy (ART) was coded as a dichotomous variable (receiving therapy or not receiving therapy). Of the 143 subjects taking ART, 91 (63.6%) were taking combination therapy without a protease inhibitor, 51 (35.7%) were taking combination therapy with a protease inhibitor, and 1 (0.7%) was taking monotherapy (not a protease inhibitor). Subjects generally reported "good" compliance with the ART regimen. Of the 143 subjects taking ART, data on compliance were available for 132 (92.3%). Of these 132 subjects, 13 reported taking the drugs "not at all"or "once in a while"(9.8%), 11 reported taking them "half the time" (8.3%), 54 reported taking them "most of the time"(40.9%), and 54 reported taking them "all of the time" (40.9%).

Demographic, Health, and Behavior Characteristics

The original REACH ethnicity/race variable included six categories: Hispanic black, white, or other and non-Hispanic black, white, or other. In regression analysis, this single variable was divided into separate, dichotomous variables for race (black or not black) and ethnicity (Hispanic or not Hispanic). Current pregnancies were noted at each visit as a dichotomous variable. Subjects were asked if they currently smoked cigarettes at each visit, and this information was recorded as a dichotomous variable. Consumption of alcohol, use of marijuana, and use of other illicit drugs in the preceding three months were reported separately and used to determine prevalence and intensity of use. For all three categories (alcohol, marijuana, and other illicit drugs), subjects reported whether they had consumed any alcoholic beverage or used marijuana or illicit drugs (yes = 1 or no = 0) and the intensity of use (0 = zero times, 1 = once a month or less, 2 = more than once a month but less than once a week, 3 = once or more times a week but not every day, or 4 = every day). The illicit drugs category included cocaine, crack cocaine, methamphetamines, amphetamines, hallucinogens, and inhalants (eg, solvents).

Immune Activation Data Collected for the Present Study

Blood Collection and Processing

Site-to-site variation within biochemical variables was minimized by providing all sites with the same blood collection and processing tubes from a central source and by processing and analyzing all samples collected for this study in batch at a central laboratory. Nonfasting blood was collected at a regularly scheduled REACH study visit into 2 royal blue–topped 7-mL heparinized Vacutainer tubes (product no. 369736; Becton-Dickinson, Franklin Lakes, NJ) for trace element analysis. Tubes were immediately wrapped in aluminum foil to protect them from light and were placed on ice. Further processing was performed within 4 hours. Blood was centrifuged at 4°C for 10 minutes at 1500 *g* to separate plasma, which was aliquoted into snap-cap polypropylene microcentrifuge tubes. After processing, all samples were frozen at −20°C or −70°C for up to 1 month before being shipped to the University of California at Davis on dry ice by overnight courier, where they were frozen at −70°C.

Immune Activation Variables

C-reactive protein (CRP) was measured on an Immulite 1 autoanalyzer using the High Sensitivity CRP kit (Diagnostic Products Corporation, Los Angeles, CA). Ceruloplasmin was measured on a Hitachi 902 Autoanalyzer using the manufacturer's reagent kit (Roche/Hitachi, Indianapolis, IN). Neopterin was measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (American Laboratory Products Company, Windham, NH).

Oxidative Damage Variables

Total plasma malondialdehyde was measured by high-performance liquid chromatography (HPLC) determination of the thiobarbituric acid–malondialdehyde adduct (TBA-MDA).[10] Plasma protein carbonyls were determined by ELISA.[11]

Statistical Analysis

Overview

In our plan for this cross-sectional analysis, we included 4 types of variables: (1) demographic, health status, and

behavior (age, gender, pregnancy status, BMI, race, ethnicity, smoking, alcohol consumption, marijuana use, and illicit drug use); (2) HIV status and disease severity (HIV status, $CD4^+$ T-cell count, plasma virus load, use of ART); (3) immune activation (activated $CD8^+$ T cells, a proxy for HIV-specific cytotoxic T cells; neutrophils; plasma neopterin, a product of activated macrophages; and 2 acute-phase proteins, CRP and ceruloplasmin); and (4) oxidative damage (plasma protein carbonyls and plasma malondialdehyde). Our principal hypothesis predicted that HIV infection and disease severity would increase immune activation and thus increase oxidative damage. We also predicted that demographic (eg, gender), health status (eg, BMI) and behavior (eg, marijuana use) variables might modify these associations.

Composite Variables

Three new variables were created using oxidative damage measures. These were PC90, MDA90, and OX90. These dichotomous variables divided subjects into those above and below the 90th percentile for protein carbonyl concentration (≥0.136 nmol/mg of total protein), for malondialdehyde concentration (≥0.313 μmol/L), or for either measure, respectively. Because malondialdehyde concentrations differed by gender, we examined these distributions separately; the cutoff value was the same regardless of whether subjects were separated by gender or grouped together.

Study Site Variables

Because there were 14 participating REACH sites (from a total of 16 original REACH sites), we created 13 variables (0, 1) to represent the sites. The 14 participating sites were Children's Hospital, Birmingham; Children's Hospital of Los Angeles, Los Angeles; Children's Hospital National Medical Center, Washington, DC; Children's Hospital of Philadelphia, Philadelphia; Cook County Hospital, Chicago; Montefiore Medical Center, Bronx; Mount Sinai Medical Center, New York; Tulane Medical Center, New Orleans; University of Maryland, Baltimore; University of Miami/Jackson Memorial Medical Center, Miami; Children's Diagnostic and Treatment Center, Fort Lauderdale; University of Medicine and Dentistry of New Jersey, Newark; State University of New York at Brooklyn, Brooklyn; and St. Jude Children's Research Hospital, Memphis.

Categoric Analysis

Categoric analysis was performed using unpaired Student *t* tests, 1-way ANOVA, and 2-way ANOVA. Subjects were grouped by HIV status, stage of HIV disease as indicated by $CD4^+$ T-lymphocyte count, use of ART, and gender for these analyses. These analyses were performed using Sigma-Stat for Windows, version 2.03 (Jandel Scientific, San Rafael, CA). Unless otherwise indicated, data are presented as mean ± SD.

Regression Analysis

Initial analysis of associations among continuous variables was done using Pearson or Spearman rank-order correlation analysis. Associations among categoric variables were analyzed using logistic regression analysis. The association of demographic, health status, and behavior variables; HIV variables; and immune activation variables with oxidative damage was examined using backward multiple regression analysis. A probability value of 0.05 was used for inclusion of terms in the resultant model. Because significant variation was seen among sites in the markers of oxidative damage, study site was included in all models as a fixed effect. Gender was also included as a fixed effect, because multiple interactions were seen in categoric and regression analysis between gender and markers of immune activation. Gender interaction terms were also evaluated as indicated (eg, gender × $\log_{10}$ ceruloplasmin concentration).

RESULTS

Demographic and Health Variables in HIV-Positive and HIV-Negative Subjects

Summary data for demographic, health status, and behavior variables are shown in Table 1. HIV-negative subjects were slightly older and reported lower alcohol intake than HIV-positive subjects. In addition, male HIV-positive subjects had a lower median BMI than did male HIV-negative subjects. Body weight did not differ by HIV status for either gender (data not shown). Seventy-three percent (191 of 262) of HIV-positive subjects reported their race as black compared with 64% (81 of 126) of HIV-negative subjects ($P = 0.11$). Nineteen percent (50 of 262) of HIV-positive subjects and 23% (29 of 126) of HIV-negative subjects reported their ethnicity as Hispanic ($P = 0.44$).

Multiple logistic regression analysis was used to characterize the associations among the 4 behavior variables: smoking, alcohol consumption, marijuana use, and illicit drug use. Among smokers, an odds ratio (OR) significantly greater than 1.0 was found for marijuana use and illicit drug use (OR = 4.31, $P < 0.001$; OR = 3.35, $P = 0.011$, respectively). Among those reporting alcohol consumption, significant ORs were also seen for marijuana use and illicit drug use (OR = 5.60, $P < 0.001$; OR = 5.38, $P = 0.004$, respectively). Among marijuana users, significant ORs were seen for smoking and alcohol use (OR = 4.31, $P < 0.001$; OR = 5.62, $P < 0.001$, respectively), as was also true for illicit drug users (OR = 3.32, $P = 0.013$; OR = 5.30, $P = 0.004$, respectively). Thus, significant associations were seen among these behavior variables, with the exceptions that smoking and alcohol use were not significantly associated with each other and illicit drug use and marijuana use were not associated with each other.

Male participants were more likely than female participants to report consumption of alcohol (58% [53 of 91] vs. 41% [117 of 283]; $P = 0.007$) and use of illicit drugs (13% [12 of 91] vs. 5.6% [16 of 284]; $P = 0.031$), but the intensities of use did not differ between genders. The rate of reported marijuana use did not differ by gender (32% [29 of 91] vs. 27% [76 of 284]; $P = 0.42$), but the intensity of use reported by male participants (3.3 ± 0.8) was greater than that reported by female participants (2.7 ± 1.2; $P = 0.018$).

Demographic, Health, and HIV Variables: Associations With Stage of Disease

HIV-positive subjects were grouped by stage of disease using $CD4^+$ T-cell categories of <200, 200 through 499, and

TABLE 1. Comparison of Demographic, Health Status, and Behavior Variables Between HIV-Positive and HIV-Negative Subjects

Variable % or Mean ± SD (n/Total or n)	HIV-Positive	HIV-Negative	P*
Gender, % female	74% (197/265)	77% (98/127)	0.63
Age (y)	20.0 ± 1.6 (265)	19.4 ± 1.5 (127)	<0.001
Age range (y)	13.8–23.2	14.8–22.9	
Ethnicity and race			
% Non-Hispanic black	70.6% (185/262)	60.3% (76/126)	0.31
% Non-Hispanic white	3.1% (8)	6.3% (8)	
% Non-Hispanic other	7.3% (19)	10.3% (13)	
% Hispanic black	2.3% (6)	4.0% (5)	
% Hispanic white	3.8% (10)	3.2% (4)	
% Hispanic other	13.0% (34)	15.9% (20)	
Current smokers	42% (111/263)	35% (44/127)	0.19
Consuming alcohol (previous 3 mo)	41% (103/250)	54% (67/124)	0.025
Intensity of use†	1.71 ± 0.82	2.02 ± 0.90	0.023
Using marijuana (previous 3 mo)	25% (63/251)	34% (42/124)	0.097
Intensity of use†	2.90 ± 1.09	2.81 ± 1.13	0.67
Using illicit drugs (previous 3 mo)	7% (18/251)	8% (10/124)	0.92
Intensity of use†	1.83 ± 0.98	1.60 ± 1.08	0.57
Currently pregnant, % of female subjects	9.4% (18/192)	11.5% (11/96)	0.73
BMI (kg/m^2)‡			
Female	27.5; 23.4, 33.8 (195)	28.0; 22.3, 34.2 (98)	0.81
Male	22.2; 19.9, 24.1 (68)	23.2; 21.5, 27.7 (29)	0.033

*Comparisons by Student t test or χ^2 test.
†Intensity definitions: 1, once a month; 2, once a month but < once a week; 3 > once a week but < daily; 4, daily.
‡BMI values are median; 25th, 75th percentiles. Comparisons were made using the Mann-Whitney rank-sum test. Comparison of nonpregnant female subjects gave essentially identical results.

500 cells/mm^3. The distribution of HIV-positive subjects in each category was 13%, 38%, and 49%, respectively (n = 263). The percentage of subjects in each category did not differ by gender (P = 0.15). Among the HIV-negative subjects, the percentage of subjects in each CD4$^+$ T-cell category was 0%, 8%, and 92%, respectively (n = 125; $P < 0.001$ compared with HIV-positive subjects). As seen in Table 2, the percentage of subjects with clinical indicators of intermediate or late HIV disease was progressively greater in those with lower CD4$^+$ T-cell counts. The same was true of plasma virus load. No differences were seen among the 3 CD4$^+$ T-cell categories in age, gender, race, Hispanic ethnicity, BMI, smoking, alcohol consumption, marijuana use, or illicit drug use. Although BMI did not differ in this categoric analysis, regression analysis showed a significant positive association between CD4$^+$ T-cell count and BMI for HIV-positive female participants (R^2 = 0.042, P = 0.004, n = 195) but not for HIV-positive male participants (R^2 = 0.023, P = 0.22, n = 68).

Immune Activation: Associations With HIV Infection and Stage of Disease

Correlation Among Immune Activation Markers

Five measures of "immune activation" were used in this study: neutrophil count, 2 acute-phase proteins (CRP and ceruloplasmin), a marker of macrophage activation (neopterin), and "activated" CD8$^+$ T cells. Within this set of variables, $\log_{10}$ neutrophil count correlated positively with the 2 acute-phase

TABLE 2. Comparison of Virus Load, Use of ART, and Clinical Progression Among HIV-Positive Subjects Grouped by CD4$^+$ T-Cell Count

	Peripheral Blood CD4$^+$ T Cells/mm^3			
Variable % or Mean ± SD (n/Total or n)	<200	200–499	≥500	P
Plasma virus load ($\log_{10}$ copies/mL)	4.91 ± 1.08 (32)*	3.96 ± 0.72 (81)*	3.44 ± 0.76 (88)*	<0.001
Use of ART	47% (16/34)	55% (57/103)	55% (70/128)	0.68
CDC clinical progression categories				
Early disease	32% (11)	50% (52)	65% (83)	0.002
Intermediate or late disease	68% (23)	50% (51)	35% (45)	

*Means are significantly different at $P < 0.05$ using all pairwise comparison procedures with Bonferroni correction after 1-way ANOVA or Kruskill-Wallace nonparametric ANOVA.

proteins, $\log_{10}$ CRP (R = 0.240, $P < 0.001$, n = 374), and $\log_{10}$ ceruloplasmin (R = 0.123, $P = 0.017$, n = 374) but negatively with the 2 HIV-related markers of immune activation, $\log_{10}$ neopterin (R = −0.288, $P < 0.001$, n = 370) and $\log_{10}$-activated $CD8^+$ T-cell count (R = −0.176, $P < 0.001$, n = 383). The 2 acute-phase proteins correlated positively with each other (R = 0.517, $P < 0.001$, n = 375) but not with the HIV-related markers, whereas the 2 HIV-related markers correlated positively with each other (R = 0.367, $P < 0.001$, n = 369) but not with the acute-phase proteins.

Association of Immune Activation Markers With HIV Infection and Stage of Disease

Summary data for the 5 immune activation markers are shown for HIV-positive and HIV-negative subjects in Table 3. Mean values for activated $CD8^+$ T cells and neopterin concentration did not differ by gender, but both markers were significantly elevated in HIV-positive subjects. The mean neopterin concentration ($\log_{10}$ μg/L) in subjects with $CD4^+$ T-cell counts <200, 200 through 499, and ≥500 cells/mm^3 were 1.008 ± 0.253 (n = 34), 0.870 ± 0.219 (n = 96), and 0.775 ± 0.228 (n = 123), respectively (all means different from each other at $P \leq 0.008$). Thus, neopterin concentration was progressively higher in those with more advanced disease. Activated $CD8^+$ T-cell counts did not differ by stage of disease.

Neutrophil counts were lower in subjects with HIV infection (see Table 3) and did not differ by gender. The mean neutrophil count ($\log_{10}$ mm^3) in subjects with $CD4^+$ T-cell counts <200, 200 through 499, and ≥500 mm^3 were 3.279 ± 0.230 (n = 34), 3.350 ± 0.240 (n = 102), and 3.443 ± 0.214 (n = 125), respectively. The mean neutrophil counts did not differ between the 2 lowest CD4 categories ($P = 0.34$), but the mean neutrophil counts in those with CD4 T-cell counts <200 and from 200 through 499 cells/mm^3 were both lower ($P = 0.002$ and $P = 0.019$, respectively) than the mean for those with a $CD4^+$ T-cell count ≥500 cells/mm^3.

Log_{10} CRP concentrations were higher in female participants than in male participants (0.213 ± 0.0404 vs. −0.0814 ± 0.0723 μg/L; $P < 0.001$) but did not differ by HIV status ($P = 0.22$ by 2-way ANOVA). There was an interaction of marginal statistical significance ($P = 0.055$) between gender and HIV status, however, with CRP concentrations tending to be higher in HIV-positive male participants than in HIV-negative male participants, with the opposite being true in female participants (see Table 3). When CRP levels were compared among subjects grouped by gender and HIV status/$CD4^+$ T-cell count (data not shown), this interaction was significant ($P = 0.014$). Although CRP concentrations did not differ by HIV status/$CD4^+$ T-cell count in female participants, there was a significant trend toward higher CRP levels in HIV-positive male participants with low $CD4^+$ T-cell counts, although none of the direct comparisons between CD4 T-cell groups were statistically significant.

Log_{10} ceruloplasmin concentrations were higher in female participants than in male participants (1.58 ± 0.0061 vs. 1.48 ± 0.011 mg per 100 mL; $P < 0.001$) and were also higher in HIV-positive subjects than in HIV-negative subjects (1.54 ± 0.0069 vs. 1.52 ± 0.010 mg per 100 mL; $P = 0.022$). As with CRP, there was a gender × HIV status interaction of marginal statistical significance ($P = 0.052$). Log_{10} ceruloplasmin concentrations did not differ by HIV status in female participants, but HIV-positive male participants had higher concentrations than HIV-negative male participants (see Table 3). No differences were seen among the $CD4^+$ T-cell categories in HIV-positive subjects for either gender.

Antiretroviral Therapy and Immune Activation

We expected that use of ART would be associated with lower plasma virus load and lower levels of HIV-related immune activation markers. Consistent with this hypothesis, we found that the mean number of activated $CD8^+$ T cells ($\log_{10}$ mm^3) was lower in HIV-positive subjects taking ART (1.554 ± 0.521, n = 141) than in HIV-positive subjects not taking ART (1.700 ± 0.568, n = 121; $P = 0.031$). Although plasma virus load was lower in subjects taking ART (3.76 ± 0.99, n = 94) than in those not taking ART (4.01 ± 0.90, n = 109), the difference was not statistically significant ($P = 0.056$). $CD4^+$ T-cell counts, neutrophil counts, neopterin concentration, and CRP concentration did not differ by ART use.

Unexpectedly, the mean (±SE) ceruloplasmin concentration ($\log_{10}$ mg/dL) was higher for those taking ART (1.560 ± 0.00882, n = 140) than for those not taking therapy (1.525 ± 0.00978, n = 114; $P = 0.008$ by 2-way ANOVA, including gender). In this analysis, ceruloplasmin concentrations were also higher in female participants than in male participants ($P <$ 0.001), as noted previously (see Table 3 and accompanying

TABLE 3. Comparison of Immune Activation Markers Between HIV-Positive and HIV-Negative Subjects

Variable Mean ± SD (n)	HIV-Positive Subjects	HIV-Negative Subjects	*P*
Activated $CD8^+$ T cells ($\log_{10}$ cells/mL)	1.622 ± 0.547 (262)	0.983 ± 0.533 (125)	<0.001
Neopterin ($\log_{10}$ μg/L)	0.842 ± 0.241 (253)	0.637 ± 0.232 (121)	<0.001
Neutrophils ($\log_{10}$ mm^3)	3.380 ± 0.232 (261)	3.558 ± 0.206 (127)	<0.001
CRP ($\log_{10}$ μg/L)			
Female	0.183 ± 0.639 (188)	0.243 ± 0.689 (95)	0.47
Male	0.048 ± 0.612 (67)	−0.211 ± 0.559 (28)	0.057
Ceruloplasmin ($\log_{10}$ mg/dL)			
Female	1.584 ± 0.097 (187)	1.579 ± 0.111 (96)	0.73
Male	1.504 ± 0.080 (67)	1.451 ± 0.0078 (28)	0.004

text). When subjects were divided into three groups—HIV-negative, HIV-positive not taking ART, and HIV-positive taking ART—and analyzed by 2-way ANOVA (including gender), the mean (±SE) plasma ceruloplasmin concentration ($\log_{10}$ mg/dL) was no greater in HIV-positive subjects not taking ART (1.525 ± 0.0102) than in HIV-negative subjects (1.515 ± 0.0103; $P = 1.0$). The mean concentration for HIV-positive subjects taking ART (1.560 ± 0.00923) was greater than the mean for HIV-positive subjects not taking therapy ($P = 0.032$) or for HIV-negative subjects ($P = 0.004$), however. Again, concentrations were higher for female participants than for male participants ($P < 0.001$) in all three groups (interaction term P value = 0.14).

Oxidative Damage: Association With Stage of Disease

Protein Carbonyls and Malondialdehyde are Independent Markers of Oxidative Damage

Plasma protein carbonyl and malondialdehyde concentrations did not correlate with each other (n = 372; $P = 0.70$, Pearson correlation coefficient) as would be expected of variables measuring the same underlying phenomenon. There was also not a significant association when the dichotomous variables (those above and below the 90th percentile for each marker) were compared by χ^2 analysis (n = 363; $P = 0.84$). Of 66 subjects in the 90th or greater percentile for either malondialdehyde or protein carbonyls, only three were in the 90th or greater percentile on both tests, as shown in Figure 1.

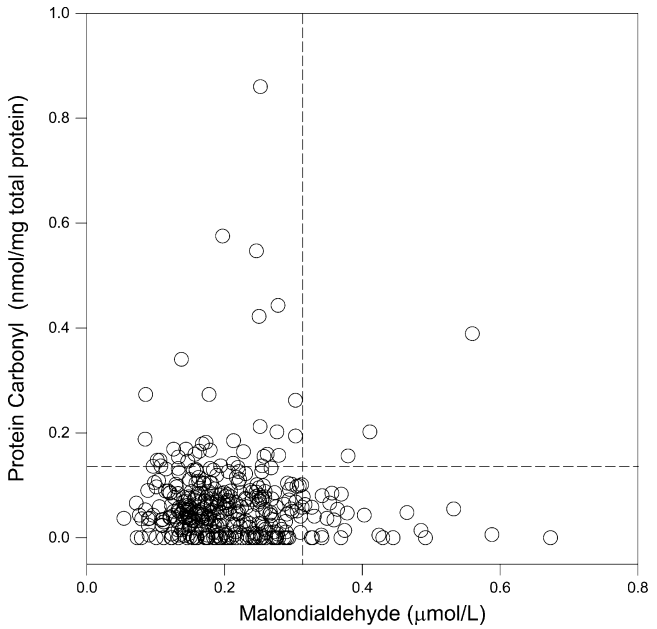

FIGURE 1. Plot of plasma malondialdehyde versus plasma protein carbonyl concentration in all subjects for whom both values were available (n = 372). Subjects to the right of the vertical dashed line have malondialdehyde concentrations greater than or equal to the 90th percentile (0.313 μmol/L). Subjects above the horizontal dashed line have protein carbonyl concentrations greater than or equal to the 90th percentile (0.136 nmol/mg of total protein).

This suggests that these two variables are measuring oxidative damage that results from different processes.

Oxidative Damage Greater in Male Participants But Not Different by HIV Status or Stage of Disease

Protein carbonyl concentration did not differ by gender nor was the mean concentration in HIV-positive subjects (0.0655 ± 0.0814 ng/mg of total protein) different from that of HIV-negative subjects (0.0633 ± 0.0862 ng/mg of total protein; $P = 0.92$). Malondialdehyde concentrations were greater in male participants (0.231 ± 0.090 μmol/L, n = 97) than in female participants (0.209 ± 0.089 μmol/L, n = 293; $P = 0.015$, analysis used $\log_{10}$ values), but mean concentrations again did not differ between HIV-positive subjects (0.224 ± 0.094 μmol/L) and HIV-negative subjects (0.211 ± 0.106 μmol/L; $P = 0.30$, 2-way ANOVA, $df = 389$). Protein carbonyl or malondialdehyde concentrations also did not differ by HIV status or $CD4^+$ T-cell count when either was examined as a continuous or dichotomous (<90th percentile vs. ≥90th percentile) variable (Table 4).

Predictors of Oxidative Damage: Identification by Multiple Regression Analysis

Smoking, Antiretroviral Therapy, and Ceruloplasmin Predict Malondialdehyde in HIV-Positive Subjects

Backward stepwise regression analysis was used to identify significant predictors of plasma malondialdehyde concentration, as shown in Table 5. When all subjects were analyzed together, only female gender was found to be a significant negative predictor of plasma malondialdehyde concentration. When HIV-positive subjects were analyzed separately, smoking was identified as a positive predictor of plasma malondialdehyde concentration. Cigarette smoking is known to be associated with increased oxidative damage,[12] so this association was not unexpected. This analysis also found that ceruloplasmin was a significant negative predictor of plasma malondialdehyde for HIV-positive subjects. In addition, an interaction of ceruloplasmin concentration with ART use was discovered and is represented in the model by the ART × ceruloplasmin interaction term (see Table 5). The positive coefficient for the interaction term indicates that the negative association of ceruloplasmin with malondialdehyde was not found (or at least the slope of the regression line was less steep) in subjects using ART.

Association of Ceruloplasmin With Lower Malondialdehyde Not Seen in Subjects Taking Antiretroviral Therapy

To clarify the associations of ART and ceruloplasmin with malondialdehyde concentration in HIV-positive subjects, the relations among these variables were examined graphically (Fig. 2). Plasma ceruloplasmin has a significant negative association (ie, slope of the regression line was significantly <0) with plasma malondialdehyde in HIV-positive subjects not taking ART (n = 106, $R^2 = 0.125$, $P < 0.001$; regression coefficient ± SE = −0.637 ± 0.159; see Fig. 2, left panel), but no significant association (ie, slope does not differ from 0) was

TABLE 4. Comparison of Oxidative Damage Measures Between HIV-Negative Subjects and HIV-Positive Subjects Grouped by $CD4^+$ T-Cell Count

Variable Mean ± SD (n)	HIV+ Subjects Grouped by CD4+ T-Cell Count (mm^3)			HIV-Negative Subjects	*P*
	<200	200–499	≥500		
PC nmol/mg of total protein	0.058 ± 0.047 (33)	0.077 ± 0.11 (97)	0.058 ± 0.057 (123)	0.066 ± 0.086 (120)	0.38
MDA μmol/L					
Female	0.202 ± 0.060 (21)	0.222 ± 0.094 (76)	0.210 ± 0.097 (100)	0.200 ± 0.082 (96)	0.49*
Male	0.243 ± 0.105 (13)	0.231 ± 0.088 (27)	0.235 ± 0.103 (28)	0.221 ± 0.073 (29)	0.94*
%PC > 90th percentile†	9.1% (3/33)	11.3% (11/97)	7.3% (9/123)	11.7% (14/120)	0.66
%MDA > 90th percentile†	8.8% (3/34)	13.6% (14/103)	10.2% (13/128)	7.2% (9/125)	0.45
%PC or MDA > 90th percentile†	18.2% (6/33)	23.8% (24/101)	17.5% (22/126)	17.4% (21/121)	0.60

*Comparisons made on $\log_{10}$ values.
†All subjects (HIV-positive and HIV-negative) were ranked by PC or MDA values, and the percentages greater than the 90th percentile are shown here.
PC indicates protein carbonyls.

seen in subjects taking ART (n = 137, $R^2 = 0.00248$, $P = 0.56$; coefficient ± SE = −0.0806 ± 0.138; see Fig. 2, middle panel). The slopes of these 2 regression lines differed significantly from each other ($P = 0.009$, Student *t* test). As a result of this difference, the mean $\log_{10}$ malondialdehyde concentration in subjects with elevated ceruloplasmin (ie, those with concentrations above the median for HIV-positive subjects) was lower for subjects not taking ART (mean ± SE = −0.799 ± 0.0234, geometric mean = 0.159 μmol/L, n = 48; $P = 0.001$) than it was for subjects taking ART (−0.702 ± 0.0182, 0.199 μmol/L, n = 79; see Fig. 2, right panel).

Malondialdehyde in HIV-Negative Subjects

Neutrophil count was negatively associated with plasma malondialdehyde, whereas the activated $CD8^+$ T-cell count was positively associated with plasma malondialdehyde (see Table 5).

Predictors of Protein Carbonyl Concentration

Significant predictors of protein carbonyl concentration were seen only in HIV-positive subjects. The activated $CD8^+$ T-cell count, CRP concentration, and intensity of marijuana use were all associated with increased oxidative damage (Table 6).

DISCUSSION

The REACH cohort fills a demographic gap in our knowledge of HIV infection. Previous studies focusing on oxidative damage as a pathogenic feature of HIV infection have been conducted in adults with mean ages >30[13–23] or in preadolescent children.[24–27] Our subjects were all between 14 and 23 years of age and were not infected by vertical transmission. In addition, participants in previous studies were predominantly male. The REACH subjects were predominantly female. Although these previous studies were done in a variety of countries, including Australia, Brazil, Canada, France, Spain, and the United States, race and ethnicity were not usually reported. Two thirds of the REACH subjects reported their race as black, and one fifth reported their ethnicity as Hispanic.

HIV-positive subjects in the REACH cohort showed a broad range of disease severity and HIV-specific immune activation. Subjects were grouped by $CD4^+$ T-cell count to

TABLE 5. Prediction of Plasma Malondialdehyde Concentration ($\log_{10}$ μmol/L) in HIV-Positive and HIV-Negative Subjects Using Demographic, Health Status, Behavioral, Disease Severity, and Immune Activation Variables in Backward Stepwise Regression Analysis

Variable*	All Subjects ($R^2 = 0.167$, n = 344)		HIV-Positive Subjects ($R^2 = 0.260$, n = 230)		HIV-Negative Subjects ($R^2 = 0.307$, n = 114)	
	Std Coef	*P*	Std Coef	*P*	Std Coef	*P*
Female	−0.161	0.003	−0.107	0.119	−0.0883	0.352
Smoking			0.137	0.035		
$\log_{10}$ ceruloplasmin			−0.332	<0.001		
Taking ART	—	—	−2.464	0.009	—	—
ART × $\log_{10}$ ceruloplasmin	—	—	2.703	0.005	—	—
$\log_{10}$ neutrophils					−0.256	0.008
$\log_{10}$ activated $CD8^+$ T cells					0.186	0.044

Study site variables and gender were included in all models, as described in Materials and Methods section. Standardized coefficients and probability values are shown for each variable. Empty cells indicate variables that were not significant, and cells with dashes (—) indicate variables not used in a particular analysis.
*Variables used in analysis: study site, age, gender, pregnancy (used only in analysis with all female subjects), BMI, race, Hispanic ethnicity, smoking, alcohol use, marijuana use, illicit drug use, HIV status, $CD4^+$ T-cell count, plasma virus load (used with HIV-positive only), neutrophils, activated $CD8^+$ T cells, CRP, and neopterin.
Std Coef indicates standard coefficient.

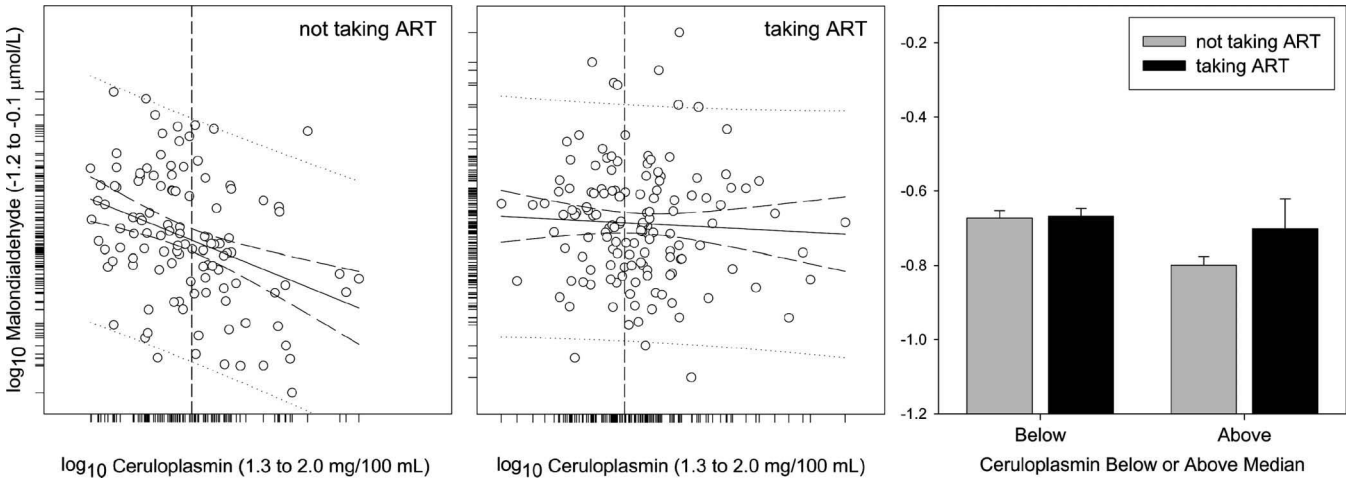

FIGURE 2. Association of plasma ceruloplasmin and antiretroviral therapy (ART) use with plasma malondialdehyde concentration in HIV-positive subjects not taking and not taking ART. Scatter plot, regression line, and 95% confidence interval for regression are shown in subjects taking (left panel) and taking (middle panel) ART. The vertical dashed line in these 2 panels indicates the median ceruloplasmin determined for all HIV-positive subjects. Mean ± SE malondialdehyde concentrations (right panel) in HIV-positive and HIV-negative subjects above and below the median ceruloplasmin concentration are shown. Statistical analysis is reported in the Results section.

reflect disease severity. Of those with $CD4^+$ T-cell counts <200 cells/mm^3 (13% of HIV-positive subjects), 68% had intermediate or late disease (using CDC clinical criteria) and the mean plasma virus load was $\log_{10}$ 4.9 copies/mL. Half of the subjects with $CD4^+$ T-cell counts from 200 to 499 cells/mm^3 (39% of HIV-positive subjects) had intermediate or late disease, and their mean plasma virus load was 10-fold lower. A minority (35%) of those with high $CD4^+$ T-cell counts (>500 cells/mm^3, 48% of HIV-positive subjects) had intermediate or late disease, and their mean virus load was $\log_{10}$ 3.4 copies/mL. Neopterin concentrations reflect macrophage activation and are thus a good marker of antiviral responses.[28] Neopterin concentrations correlated inversely with $CD4^+$ T-cell count (data not shown), as expected. Thus, $CD4^+$ T-cell count accurately reflected stage of disease in this cohort.

HIV infection was not associated with a marked induction of the acute-phase response in REACH subjects. In male participants, CRP and ceruloplasmin concentrations tended to be higher in more severe disease, but this association was not seen in female participants. Elevated CRP concentrations have been reported in more severe HIV disease in men[29] and women,[30] presumably related to coinfection with other pathogens that induce CRP. The weak association of disease severity with CRP and ceruloplasmin may be explained by a low prevalence of such coinfections. In addition, the high BMI of female subjects may be a factor. BMI had a strong, positive association with CRP (data not shown), and female REACH cohort members with $CD4^+$ T-cell counts <200 cells/mm^3 had a relatively high mean BMI at 26 kg/m^2, although it was lower than that of subjects with less severe disease ($CD4^+$ T-cell >499 cells/mm^3).

Ceruloplasmin but not CRP was higher in subjects taking ART. An increase in CRP concentrations has been observed previously after initiation of highly active antiretroviral therapy (HAART), even though plasma virus loads decreased and $CD4^+$ T-cell counts increased.[29] It is not clear why

TABLE 6. Prediction of Plasma Protein Carbonyl (ng/mg of Total Protein) in HIV-Positive and HIV-Negative Subjects Using Demographic, Health Status, Disease Severity, and Immune Activation Variables in Backward Stepwise Regression Analysis

	All Subjects (R^2 = 0.080; n = 343)		All HIV-Positive Subjects (R^2 = 0.187; n = 229)		All HIV-Negative Subjects (R^2 = 0.064; n = 114)	
Variable*	***P***	**Std Coef**	**Std Coef**	***P***	**Std Coef**	***P***
Female	−0.0683	0.228	−0.109	0.105	−0.0330	0.760
Marijuana use, intensity			0.138	0.035		
Log_{10} activated $CD8^+$ T cells			0.129	0.044		
Log_{10} CRP			0.134	0.037		

Study site variables and gender were included as fixed effects, as described in Materials and Methods section. Standardized coefficients and probability values are shown for each variable. Empty cells indicate variables that were not significant, and cells with dashes (—) indicate variables not used in a particular analysis.

*Variables used in analysis: study site, age, gender, pregnancy (used only in analysis with all female subjects), BMI, race, Hispanic ethnicity, smoking, alcohol use, marijuana use, illicit drug use, HIV status, CD4+ T-cell count, plasma virus load (used with HIV-positive only), neutrophils, activated $CD8^+$ T cells, CRP, and neopterin.

Std Coef indicates standard coefficient.

ceruloplasmin was elevated in subjects receiving ART in the REACH cohort. HIV may suppress the acute-phase response, and ART allows normalization of this response to other infections. In contrast, the level of activated $CD8^+$ T cells is lower in those on ART, as would be expected, because of decreased viral replication and a decrease in stimulation of the cytotoxic T-lymphocyte response.

A principal finding of our study is that HIV-positive subjects from this population did not have higher plasma concentrations of oxidative damage measures (malondialdehyde and protein carbonyls), than did HIV-negative control subjects of similar age, gender, and race/ethnicity. Our study is the first report of plasma protein carbonyl concentrations during HIV infection, although higher protein carbonyl concentrations were seen in activated lymphocytes isolated from HIV patients versus uninfected controls.[31] Several previous studies have also examined serum or plasma malondialdehyde by HPLC as a marker of oxidative damage. Higher levels have been seen in HIV-positive children by a group in Spain.[24,25,32] Higher levels have also been seen in Swedish men,[21] but 2 recent studies from Poland[33] and Canada[16] report no association of HIV infection with plasma malondialdehyde concentrations in adults.

Of nine studies that used less specific methods to measure lipid peroxides (primarily the thiobarbituric acid–reducing substances [TBARS] assay), eight showed higher peroxide levels in adults[15,17–20,22,34] and children[26] with HIV, whereas one study in adults showed no difference.[23] The HPLC method has the advantage of measuring a specific TBA-MDA, whereas the other methods measure a variety of reactive compounds in addition to malondialdehyde.[1] These data suggest that the more specific HPLC assay is less likely to show a difference in oxidative damage products than are less specific methods, raising the question of how meaningful the results of these other assays are in reflecting in vivo oxidative stress during HIV infection.

Multiple regression analysis revealed that predictors of malondialdehyde concentration differed between HIV-positive and HIV-negative subjects. Two principal observations were made in HIV-positive subjects. First, ceruloplasmin concentration was negatively associated with plasma malondialdehyde in HIV-positive subjects not taking ART. To our knowledge, this association has not been made previously. Ceruloplasmin is an oxidase that can reduce oxygen to water, and aceruloplasminemia is associated with increased lipid peroxidation.[35] Thus, increased ceruloplasmin levels may be associated with decreased oxidative damage because of ceruloplasmin's ferroxidase activity. In addition, ceruloplasmin-mediated oxidation of iron plays a central role in iron homeostasis and may facilitate transfer of iron from the blood to tissues during the acute-phase response. Because iron can catalyze lipid peroxidation, this activity may also help to account for the negative association of ceruloplasmin with malondialdehyde. Two previous studies have demonstrated a positive association between plasma ceruloplasmin activity and malondialdehyde concentrations in patients with rheumatoid arthritis, however.[36,37] The reason for the different findings in HIV and rheumatoid arthritis patients is not clear. It is possible that regulation of ceruloplasmin synthesis or its efficacy in controlling oxidative damage may differ between these two conditions.

A second observation of note was that ceruloplasmin concentrations were elevated in HIV-positive subjects taking ART. In addition, the negative association of ceruloplasmin with malondialdehyde was not found in subjects taking ART as it was in other HIV-positive subjects. One interpretation of this observation is that ART somehow negates the protective association between ceruloplasmin and oxidative damage, perhaps by increasing oxidative stress. Although there is not direct evidence for this, ART may affect mitochondrial function in some subjects[38] and thus might affect levels of cellular oxidative metabolites.

In HIV-negative subjects, the count of activated $CD8^+$ T lymphocytes was positively associated with malondialdehyde, whereas the neutrophil count had a negative association. To the best of our knowledge, these are novel observations. The mechanism underlying these associations is not immediately apparent. One possibility is that subjects with an active viral infection have increased oxidative damage. Such an infection could increase the count of activated $CD8^+$ T lymphocytes and produce mild neutropenia. If this were true, however, such an association would have been expected when all subjects were analyzed together or when HIV-positive subjects were analyzed, because HIV infection itself increases activated $CD8^+$ T lymphocytes and decreases neutrophils. Thus, the explanation underlying these associations may not be as direct as suggested here, although the positive association of activated $CD8^+$ T lymphocytes with protein carbonyl concentrations in HIV-positive subjects does support the conclusion that an active antiviral response does contribute to increased oxidative damage.

The second oxidative damage marker used in our study, plasma protein carbonyls, showed no correlation with malondialdehyde. Thus, the two markers seem to be identifying different types of oxidative stress (or different deficits in antioxidant protection). In agreement with this conclusion, we have not found a correlation between ceruloplasmin or ART and protein carbonyl concentration as was seen with malondialdehyde. Elevated counts of activated $CD8^+$ T lymphocytes were associated with protein carbonyl concentrations in HIV-positive subjects, however, as was seen with malondialdehyde in HIV-negative subjects. Another significant predictor of protein carbonyl concentrations was plasma CRP, suggesting that an active acute-phase response may cause increased oxidative stress, as expected. Thus, 2 markers of immune activation—the first a marker of an antiviral response and the other a marker of the acute-phase response—were associated with increased protein carbonyl concentration. Finally, intensity of marijuana use was associated with increased plasma protein carbonyl concentrations. Although 1 study has shown an increase in oxidative damage after exposure of cultured cells to marijuana smoke in vitro and lower levels of glutathione in pulmonary macrophages from marijuana users compared with nonusers,[39] this is the first report of oxidative damage in vivo in association with marijuana use. Given the known association of cigarette smoking with oxidative damage, it is perhaps not surprising that marijuana use is also associated with oxidative damage.

In summary, the REACH cohort is unique among HIV study populations because of its age, gender, and racial and ethnic composition as well as the high prevalence of obesity and overweight among its members. Previous reports have shown that the diet quality was poor in many REACH subjects with regard to total intake of energy, sodium, and cholesterol as well as the amount and type of fat.[40] In addition, diets among REACH subjects tended to be low in zinc and vitamins A and E.[41] Unlike many previous studies, no association of HIV infection with oxidative damage was found in the REACH cohort. The relative youth of the cohort and the low percentage of subjects with advanced disease may explain this observation. Oxidative damage was observed in REACH subjects, however, particularly in association with smoking, marijuana use, and immune activation. A novel observation from this study was that elevated plasma ceruloplasmin was associated with decreased oxidative damage in HIV-positive subjects not taking ART. The lack of such a protective association in those taking ART, and the elevated oxidative damage seen in those subjects, suggests that use of ART may have abolished the antioxidant protection of ceruloplasmin. Although this conclusion is speculative, future studies examining the benefits of ART for the long-term health and nutritional status of HIV patients should consider the impact of such therapy on antioxidant protection and oxidative damage.

ACKNOWLEDGMENTS

The authors acknowledge the contributions of the investigators and staff of the Adolescent Medicine HIV/AIDS Research Network (1994–2001) and the youth who participated in the research. Participating investigators and staff are listed elsewhere [J Adolesc Health. 2001;29(Suppl):5–6]. The authors thank Alina Wettstein and Tuan Nguyen for performing assays, Leslie Woodhouse for supervising assays, and Ginny Gildengorin for assistance with regression analysis.

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